ACACIA CONSTRICTA GAINS NOVEL BENEFITS FROM ANTS WHILE MINIMIZING POTENTIAL CONFLICTS

By

E. Fleur Nicklen

RECOMMENDED:

Advisory Committee Chair

Assistant Chair, Department of Biology & Wildlife

APPROVED:

Dean, College of Natural Science and Mathematics

Dean of the Graduate School

August 3, 2006

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A

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By

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Abstract

The sum of costs and benefits in an interspecific interaction determines whether the relationship is mutualistic, neutral, or antagonistic. We investigate novel benefits *Acacia constricta* may gain from ant visitors and how *A. constricta* may minimize potential costs of ant visitation. *A. constricta* attracts ants onto its foliage and encourages nesting at its base with extrafloral nectaries. Plants with basal nests have significantly greater soil nutrients and produce twice as many seeds as plants lacking basal nests (Wagner 1997). Along side these benefits, however, ants can interfere with plant reproduction. This study tests whether augmented soil nutrients increase *A. constricta's* defenses and ant attractants. We further test mechanisms *A. constricta* may have to reduce the potential costs of ants to reproduction. We found that increased soil nutrients elevated defense mechanisms in *A. constricta* and increased extrafloral nectary number, suggesting ants that provide plants with nutrients may indirectly increase plant defense as well as participate in a feed back cycle where ants increase soil nutrients allowing plants to increase ant attractants. In addition, plants have at least two mechanisms to keeps ants separated from flowers, minimizing ant costs to reproduction.

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Introduction

All interspecific interactions fall within a continuum from mutualism to antagonism. Where on this scale an interaction lies depends on multiple costs and benefits to each interacting individual as well as the context in which the interaction occurs. Investigating all factors affecting the costs and benefits inherent in interspecific interactions gives insight into whether an interaction is positive, neutral, or negative, and how such interactions can persist.

Acacia constricta is a desert shrub that bears nectar-secreting glands, extrafloral nectaries, along its leaves. Multiple ant species feed on the plant's extrafloral nectaries, but show no evidence of defending plants (Wagner 1997). Furthermore, ants significantly reduce pollen viability upon contact (Wagner 2000), and may discourage pollinator visitation as found in other systems (Ness 2006). Yet, ants that nest at the base of *A. constricta* significantly increase soil nutrients, and plants with basal nests produce nearly twice as many seeds as plants lacking ant nests (Wagner 1997). Where on the continuum from mutualism to antagonism this relationship lies is not clear.

In this study, I investigate ways in which *Acacia constricta* may gain novel benefits from ant visitors, while minimizing potential costs from visiting ants. Specifically, I ask whether fertilizer additions on par with levels of nutrients found beneath acacias with ant nests increase plant defenses and fuel a feedback cycle whereby extrafloral nectary rewards to ants are increased. Additionally, I examine ways in which

A. constricta may temporally and spatially separate ant visitors from flowers and pollinators.

Chapter 1 Nutrient amendment increases extrafloral nectary number and chemical and physical defense in *Acacia constricta*

1.1 Abstract

Ants that nest at the base of the extrafloral nectary-bearing plant, *Acacia constricta,* add nutrients to the soil surrounding the plant (Wagner 1997). Increased access to nutrients may affect how a plant allocates resources to growth, reproduction, and defense. In this study we[1] used greenhouse experiments to test the effect of nutrient amendment and maternal seed source on the allocation of resources to growth, extrafloral nectary (EFN) production, hydrogen cyanide (HCN) release, and spine length in the shrub *Acacia constricta.* We conducted two trials: in one trial we used soil native to *A. constricta* and three fertilizer levels, and in the other trial we used standard potting soil with two fertilizer levels and increased replication within seed source to test for maternal effects. Plants growing in native soil grew taller, had longer leaves, and produced more EFNs and active EFNs per leaf when given medium fertilizer relative to no fertilizer. Plants given the highest level of fertilizer had heights, leaf lengths, and numbers of total and active EFNs intermediate to, and not significantly different from, those in the no fertilizer and intermediate fertilizer treatments. Because fertilized plants in native soil produced longer leaves, the density of EFNs per unit leaf length did not vary among fertilizer treatments.

[1] EF Nicklen and D Wagner

In the potting soil trial, plant height and leaf length did not vary significantly among fertilizer treatments, and plants given more fertilizer produced a higher density of total and actively-secreting EFNs. In young leaves, HCN release per gram of damaged leaf tissue increased in response to the highest level of nutrient amendment in native soil but remained constant across nutrient treatments in plants grown in potting soil. In potting soil, spine length increased by 20% on high nutrient plants relative to medium nutrient plants, but this difference was not significant. Maternal seed source significantly affected numbers of EFNs and active EFNs, suggesting that genetics, maternal provision, or both affect EFN expression. There were no significant maternal effects on HCN production or spine length. Our results suggest that ant nests that provide particularly rich sources of soil nutrients might enhance plant chemical defense. Furthermore, the results suggest that modest increases in soil nutrients, similar to those in an average ant nest, can increase numbers of EFNs and could lead to higher ant visitation rates.

1.2 Introduction

Ant-derived nutrients may serve as currency in mutualisms in which plants provide nesting space or food rewards to ants. Some ant-plants offer ants nesting space and in return receive nutrients from the debris ants deposit in specialized leaf, stem, or petiole chambers (Janzen 1974; Rickson 1979; Rico-Gray 1989; Gay 1993; Treseder et al. 1995; Sagers et al. 2000; Fischer et al. 2003; Solano and Dejean 2004). Because soil-dwelling ants typically elevate the concentrations of nutrients such as ammonium, nitrate, and

phosphorus near their nests (Petal 1978; Culver and Beattie 1983; Beattie 1985; Wagner 1997; Wagner et al. 1997; Eldridge and Myers 1998; MacMahon 2000), plants growing nearby can benefit from access to these nutrients. Several studies have reported higher growth or reproductive success by plants growing near ants nests (Rissing 1986; Brown and Human 1997), and ant-derived nutrients bearing distinctive chemical labels have been traced to the tissues of plants growing nearby (Wagner and Jones 2006). Though it appears that ants can benefit plants by increasing soil nutrients, few studies have investigated this phenomenon as a currency in ant-plant mutualisms (but see Wagner 1997).

Many studies have investigated the role of visiting ants in reducing herbivory (reviewed by Bentley 1977; Heil and McKey 2003). All of these studies focused on ant behavior as the mechanism by which ants defend plants: aggressive ants kill or dislodge herbivores as they move throughout the plant collecting nectar. Here we explore a novel mechanism by which ants might increase plant defense: nutrients originating from ant nests enhance a plant's ability to produce chemical and physical defenses.

In general, fertilized plants have higher growth rates and greater biomass than those with limited access to mineral nutrients (Chapin 1980, Chapin et al. 1986). Plants with greater access to nitrogen tend to invest more resources in nitrogen-based defenses relative to nutrient-limited plants (Bryant et al. 1983; Herms and Mattson 1992). For example, several studies have demonstrated that cyanide production increases with nitrogen fertilization (Dement and Mooney 1974; Forslund and Jonsson 1997; Burns et al. 2002).

The carbon-nutrient balance hypothesis predicts that carbon-based defense mechanisms will decrease with increasing mineral nutrients (Bryant et al. 1983; Herms and Mattson 1992; Stamp 2003). Because nutrient-limited plants have slow growth relative to photosynthetic rates, may carbohydrates accumulate (Wong 1973; McKey 1979; Chapin 1980; Herms and Mattson 1992). Carbohydrates in excess of what is needed for growth may be allocated to C-based defenses (Bryant 1987), such as polyphenols, terpenes, tannins, lignins, tough leaves, or spines. Given fertilizer, the ratio of carbon to nitrogen and phosphorus is predicted to decrease, reducing excess carbohydrate production, and, thus, the amount of carbon allocated to defense. Evidence supporting this prediction is often conflicting, with some carbon-based compounds decreasing with fertilizer (Iason and Hester 1993; Haukioja et al. 1998) and others remaining constant (Reichardt et al. 1991; Iason and Hester 1993; Dudt and Shure 1994; Haukioja et al. 1998). In particular, growth of spines does not follow the resource allocation predictions: spines have been found to grow in response to herbivory regardless of nutrient availability (Myers 1987), to grow denser in high nutrient sites (Pisani 1997), but to not respond to fertilizer treatments (Pisani 1997), even in the presence of herbivory (Myers 1987).

Extrafloral nectaries (EFNs), nectar-secreting glands located outside of flowers, are often considered an indirect defense mechanism: they can attract ants that defend plants from herbivores. EFNs are carbon structures that have high densities of mitochondria (Bentley 1977), which likely make the tissue expensive to produce and maintain. Because EFNs may be both carbon and nutrient limited, it is difficult to use the

carbon-nutrient balance hypothesis as a predictor; however, plants with greater access to soil nutrients may produce more EFNs than nutrient-limited plants. Extrafloral nectar, on the other hand, is primarily composed of sugar (Bentley 1977) and may not be expected to increase with increasing nutrients.

In this study, we use a greenhouse experiment to test the effect of nutrient amendment and maternal seed source on allocation to growth, EFN production, chemical defense, and spine length in *Acacia constricta*. *A. constricta* is a desert shrub that bears EFNs on its leaves and associates with ants that sometimes nest at the plants' bases, increasing soil nutrients (Wagner 1997). It has both spines and chemical defense in the form of cyanide.

1.3 Materials and methods

1.3.1 Study system and species

Acacia constricta is a deciduous shrub that ranges from western Arizona east to western Texas and south to Oaxaca Mexico at elevations of 450 to 2000 m (Gucker 2004). *A. constricta* produces leaves and inflorescences following monsoons that typically occur in July and August.

Our generalizations about *A. constricta's* ecological interactions are based on field research at a study site in southeast Arizona, USA (31°54'01" N, 109°05'26 W). *A. constricta* has multiple mechanisms for defense against herbivory. Plant tissues contain cyanogenic glucosides, which interact with β-glucosidases to release hydrogen cyanide

when cells are damaged (Seigler et al. 1976). In addition, paired spines occur at each leaf axil. The leaves of mature *A. constricta* plants bear EFNs along the rachis. The first leaves produced by *A. constricta* seedlings lack EFNs. In the greenhouse, most seedlings develop leaves with EFNs within 2 months (Wagner, unpubl. data). From then on, the vast majority of leaves have at least one EFN. On mature plants growing under natural conditions, the number of EFNs per leaf averages 2.0 (range 0 – 5, n = 155 plants, 6 leaves per plant) (Wagner, unpubl. data). EFNs are frequently visited by ants.

The most common ant visitors to *A. constricta* EFNs at this site are *Formica perpilosa, Myrmecocystus mimicus, Dorymyrmex* sp. *(smithi* complex), *and Forelius pruinosus* (pers. observation). Ants feed on insects, nectar secreted by EFNs, and exudates from tended caterpillars and homopterans. Experimental exclusion of ants from acacias has provided no evidence that visiting ants defend plants against herbivory (Wagner and Kurina 1997); however, ants may benefit plants through nutrient addition.

Colonies of *F. perpilosa* nest permanently under *Prosopis juliflora*, but form temporary, satellite nests under *A. constricta* following the summer rains. The nutrient content of soil under *A. constricta* plants with basal ant nests is significantly elevated relative to that of plants without ant nests: on average, soils under plants with ant nests contain 575% more ammonium, 167% more nitrate, and 54% more phosphorus than plants lacking ant nests (Wagner 1997). In a comparison of *A. constricta* plants with and without *F. perpilosa* nests at the base, plants with ant nests suffered similar levels of herbivory but produced about twice as many seeds as plants without nests (Wagner

1997). Colonies of *D. smithi* also occasionally nest underneath *A. constricta*, and their nest similarly elevate soil nutrients (Wagner and Nicklen, in prep.).

1.3.2 Experimental setup

1.3.2.1 Seed and soil collection

Seeds and soil were collected from the vicinity of our Arizona field site and transported to the Institute of Arctic Biology greenhouse at the University of Alaska Fairbanks (UAF). Soils were collected from spaces between shrubs and ant nests, to the same depth (10cm) as those sampled by Wagner (1997). In September 2004 we collected seeds from 25 wild *Acacia constricta* plants in the same area. To minimize relatedness among seed source plants, we chose plants separated by at least 10 meters.

Sufficient soil was available to test only the effect of nutrient amendment on plant characteristics with no replication of the maternal seed source within fertilizer treatment. To further explore the effect of genetic factors and their interaction with nutrient amendment, and to investigate the generality of plant responses across soil conditions, we conducted a second trial of the experiment using plants grown in standard potting soil.

1.3.2.2 Native soil trial

In this trial we grew plants in native soil with three nutrient treatments. In November 2004, we scarified and germinated seeds from 21 different maternal seed plants. Three seedlings from each seed source were planted separately in approximately 990g of native soil in 0.95 L styrofoam cups. Each seedling per seed source was randomly assigned one

of three amendment treatments: no fertilizer, "medium", and "high" fertilizer. The fertilizer was 14-14-14 NPK with 8.2% NH_4^+, 5.8% NO_3^-, 14% P_2O_5, and 14% K_2O) (slow release, Osmocote, Scotts Co, *city*). The amount of nutrients added is detailed in Table 1.1. Fertilizer treatments were intended to approximate the range of nutrient conditions experienced by plants under natural conditions. Since *Acacia constricta* plants can potentially form symbioses with nitrogen fixing bacteria, nitrogen may not be a limiting resource; for this reason, we chose to base fertilizer amendments on the natural phosphorus levels found by Wagner (1997) (Table 1.1). The no-fertilizer treatment was intended to roughly approximate the nutrient level of plants without basal nests. The medium-nutrient treatment was amended to approximate soil with the average nutrient level of plants with basal *F. perpilosa* nests, and the high-nutrient treatment to approximate soil with two times the standard deviation of soil nutrient levels measured under plants with basal nests (Wagner 1997). Fertilizer treatments were reapplied every 4 months. Plants were arranged in random order on the greenhouse bench and rotated 4 times during the 9 month trial. Water was delivered via a single ceramic automatic-waterer per pot (Lee Valley, Ogdensburg, NY) connected to one of 4 buckets of water; plants were randomly assigned to one of the 4 water sources.

1.3.2.3 Potting soil trial

In this trial we grew plants in a standard potting soil mixture (2 coconut husk: 1 vermiculite) under two fertilizer treatments and increased the replication within maternal seed source. In February 2005, we scarified, germinated, and planted 6 seeds from each

of 25 different mother plants. Half of the plants, selected at random, received the medium-nutrient amendments as described above, and the rest received high-nutrient amendments (Table 1.1). Plants were randomly arrayed on the greenhouse bench. We rotated plants on the bench four times during the 9 month trial. Plants were top-watered as needed and fertilizer was reapplied every 4 months.

1.3.3 Data collection and analysis

1.3.3.1 Growth characteristics

We measured plant height and rachis length on plants in native and potting soil after four and three months of growth respectively. In October 2005 we counted the total number of leaves and measured the aboveground dry biomass of the plants grown in potting soil. For a subset of the plants (two plants per seed source), we dried and weighed the course roots and root nodules. Because the plants in native soil were needed for a follow-up experiment, we did not measure biomass; once the subsequent experiment was complete, we scored the roots for presence or absence of root nodules.

1.3.3.2 Extrafloral nectary count and nectar secretion

We counted the number of EFNs on plants growing in native soil in April 2005 and those growing in potting soil in May 2005. Plants typically grew by elongating the central stem rather than branching. As a result, new leaves were located at the top of the stem and older leaves were located progressively further down the stem. For each plant, we counted the number of EFNs per leaf and measured rachis length to the nearest mm on 9

leaves, 3 new leaves from the top third of the plant, 3 leaves from the middle third, and 3 old leaves from the bottom third. Here and throughout the chapter, new leaves were unfurled, but not yet fully expanded. In addition, we observed each EFN with a 10x lens and recorded whether or not nectar was visible. We used the number of EFNs in which nectar was visible as an estimate of the number of actively secreting nectaries. All measurements were made without picking the leaves or damaging the plant.

1.3.3.3 Cyanide production

In July 2005, we collected 2 new leaves from the tips of growing shoots and 2 older leaves (at least 15cm from the growing tip) from the plants growing in native soil. In October 2005 we collected 2 new leaves and 1 older leaf from each of the plants growing in potting soil. Within a half hour of collection, each leaf was individually crushed with a micropestle in a microcentrifuge tube. We incubated each open microcentrifuge tube containing a ground leaf in a sealed scintillation vial containing a trap of 0.5ml 1M NaOH for 24 hours at room temperature (Schappert and Shore 1999). The cyanide content of the extract was measured colorimetrically following the methods of Lambert et al. (1975) and Brinker and Seigler (1989). We dried the leaves for four days and weighed them to the nearest mg. Although it is the amount of HCN that is released per unit of leaf damaged that we expect to be most relevant to herbivores, results for per leaf cyanogenesis are reported as well, in order to better explain the relationship between fertilizer treatment and leaf age.

1.3.3.4 Spine length

We measured the length of spines on the plants in potting soil in October 2005. One spine from a pair was measured. Since spines on new shoots may not be fully formed, we measured three spines at consecutive leaf nodes 7cm from the tip of the longest shoot.

1.3.3.5 Soil properties

To characterize soil particle distribution, we first passed each sample through a 2mm sieve to measure the percentage of gravel, then measured the percent sand, silt, and clay in the soil with a hydrometer (Gee and Bauder 1986). We also measured soil bulk density and porosity. Three soil replicates per soil type were used for each analysis.

We measured the standing stocks of available mineral nitrogen and phosphorus in soils prior to addition of fertilizer. Available orthophosphate was extracted in 0.03-N NH_4F and 0.025-N HCl (Bray and Kurtz 1945). Mineral nitrogen was extracted in 2M KCl. Concentrations of NH_4-N, NO_3-N, and PO_4-P were determined colorimetrically using an automated ion analyzer. Soil nutrient concentractions were adjusted for soil moisture content. We measured the pH in a 1 to 2 solution of soil and distilled water.

In addition, we determined the cation exchange capacity of each soil at pH 7 (Chapman 1965). Soils were saturated with ammonium acetate, washed, and extracted in 1M KCl. The concentration of NH_4-N in extraction was measured colorimetrically following Soloranzo (1969).

1.3.3.6 Data analysis

Data were tested for homogeneity of variances, and the residuals of statistical models were tested for normality. Data sets that did not conform to parametric assumptions were transformed.

On plants grown in native soil, the effect of nutrient amendment on the dependent variables EFNs per leaf, EFNs with visible nectar per leaf, HCN released, plant height, and rachis length was assessed using ANOVA, with fertilizer treatment as the main effect and maternal seed source as a random blocking factor. Where relevant, we included leaf age and the interaction between treatment and leaf age. For data sets with more than one sample per plant, plant was included in the model as a random factor. Means were compared using Tukey-Kramer HSD. We used a logistic model to test whether fertilizer treatments affected the presence or absence of root nodules, and t-tests to compare EFN and HCN production in plants with and without root nodules.

For plants grown in potting soil, we tested the effect of treatment, maternal seed source, and leaf position on EFNs per leaf, EFNs containing nectar per leaf, and HCN production. Plant individual was included as a random effect. Spine length and total number of leaves were examined using two-way ANOVAs with seed source and treatment as main effects and plant as a random effect (for spines only). We also tested the effect of fertilizer treatments on root, shoot, and root nodule dry biomass in one-way ANOVAs.

Differences between native soil and potting soil properties were assessed with t-tests. Data were analyzed using SAS (SAS institute 2004) with PROC MIXED for ANOVAs and PROC LOGIT for logistic models.

1.4 Results

1.4.4 Growth characteristics

In general, plants given no fertilizer were smaller and had smaller leaves than those given fertilizer. In native soil, height and leaf length were significantly increased by fertilization (Table 1.2; height: $F_{2,38} = 5.80$, $P = 0.006$, rachis: $F_{2,474} = 8.37$, $P < 0.001$), however, plants given medium and high fertilizer were similar in height and rachis length ($P > 0.05$) (Table 1.2). In potting soil, height and rachis length did not differ between medium and high fertilizer (Table 1.2; height: $F_{1,97} = 2.10$, $P = 0.15$ $F_{1,1127} = 3.32$, $P = 0.07$).

In potting soil maternal seed source explained a significant portion of the variance in growth (height: $F_{24,97} = 2.40$, $P = 0.001$, rachis: $F_{24,1127} = 1.88$, $P = 0.006$). Maternal lines responded differently to fertilizer treatments in height, (interaction $F_{24,97} = 1.94$, $P = 0.01$), but not rachis length (interaction $F_{24,1127} = 1.04$, $P = 0.41$). Of the maternal lines, one grew taller in the high fertilizer treatment, one grew taller in the medium fertilizer, and the rest had no response to treatment (Tukey-Kramer $P < 0.05$).

The total number of leaves on plants grown in potting soil did not differ between fertilizer treatments (Table 1.2; $F_{1,80} = 2.83$, $P = 0.1$), but was influenced by seed source

($F_{2,80} = 1.86$, $P = 0.02$). Plants from different seed sources responded similarly to treatment in terms of total number of leaves (interaction: $F_{24,80} = 1.08$, $P = 0.39$).

Plants grown in potting soil and given high nutrients had higher shoot and coarse root dry biomass than plants given medium fertilizer amendments (Table 1.2; Shoot: $F_{1,88} = 13.44$, $P < 0.001$, Root: $F_{1,41} = 7.59$, $P = 0.009$). All of the roots examined contained nodules. Neither the dry nodule biomass or the biomass: nodule ratio differed between fertilizer treatments (Table 1.2, nodule: $F_{1,41} = 0.52$, $P = 0.47$, biomass : nodule: $F_{1,39} = 2.66$, $P = 0.11$). Of the native soil plants, 75% contained root nodules. The presence or absence of nodules was not affected by fertilizer treatment ($X^2_{2, n=60} = 0,01$ $P = 0.99$). Within the group of plants grown in native soil, nodulated plants did not produce more EFNs or HCN than those lacking nodules (HCN: $t_{58} = 0.52$, $P = 0.61$, EFNs: $t_{59} = 0.08$, $P = 0.93$).

1.4.2 Extrafloral nectary counts and nectar secretion

1.4.2.1 EFNs per leaf

Nutrient amendment impacted EFN numbers, however, these effects were complex. For plants grown in native soil, the overall effect of nutrient amendment on the number of EFNs per leaf was not statistically significant (Fig. 1.1A; $F_{2, 480} = 2.92$, $P = 0.055$), however, contrasts revealed that plants given the medium level of nutrients had significantly more EFNs per leaf than plants given no fertilizer (Tukey-Kramer $P < 0.05$). Plants in the high nutrient treatment had intermediate numbers of EFNs and did not differ

significantly from either of the other treatments. The number of EFNs per leaf increased as rachis length increased so that EFN density (EFNs per rachis length) remained constant across fertilizer treatments ($F_{2,472} = 0.58$, $P = 0.56$). Leaf age had a strong influence on the number of EFNs per leaf ($F_{2, 480} = 80.85$, $P < 0.001$). Older leaves, collected from the bottom of the plants, had significantly fewer EFNs than both middle and young leaves (Fig. 1.1A; Tukey-Kramer $P < 0.05$). Leaves from the middle of the plant and young leaves did not differ significantly in EFN number (Tukey-Kramer $P > 0.05$). Leaf age did not interact with treatment ($F_{4,480} = 1.15$, $P = 0.33$).

For plants grown in potting soil, fertilizer treatment, leaf age, and maternal seed source all significantly influenced the number of EFNs per leaf. Plants given high fertilizer had significantly more EFNs per leaf than plants in medium fertilizer (Fig. 1.1B; $F_{1,1129} = 4.03$, $P = 0.04$). Unlike the plants grown in native soil, the density of EFNs, as well as the total number per leaf, was higher in the high fertilizer treatment ($F_{1,1160} = 11.29$, $P < 0.001$). Plants given high fertilizer had 22% more EFNs per leaf length than plants given medium fertilizer. Young leaves had the most EFNs, leaves from the middle section had significantly fewer, and old leaves had significantly fewer still (Fig.1.1B, $F_{1,1129} = 363.47$, $P < 0.001$, Tukey-Kramer $P < 0.05$). Leaves of different ages varied in the strength of their response to treatment; young and old leaves responded more strongly to treatment than leaves from the middle of the plant (Fig 1.1B; interaction: $F_{48,1129} = 2.22$, $P < 0.001$).

Maternal seed source explained a significant amount of the variation in the EFN number of plants grown in potting soil ($F_{24,1129} = 1.67$, $P = 0.02$). The response to

fertilizer treatments varied for plants from different maternal lines (interaction: $F_{24,1129} = 1.65$, $P = 0.03$). Of the maternal lines, 24% produced more EFNs per leaf when given high nutrients, one maternal line produced fewer EFNs per leaf when given high nutrients, and the rest had no response to treatment (Tukey-Kramer $P < 0.05$). Maternal seed source also significantly interacted with leaf age (interaction: $F_{2,1129} = 24.49$, $P < 0.001$).

1.4.2.2 EFNs per leaf with visible nectar

The number of active EFNs per leaf responded to fertilizer amendments in a qualitatively similar manner to the total EFNs per leaf. In native soil, fertilizer treatment significantly affected the number of EFNs containing visible nectar (Fig. 1.1C; $F_{2,474} = 4.03$, $P = 0.02$). Plants given medium fertilizer had significantly more EFNs containing nectar than both plants given either high or no fertilizer (Tukey-Kramer $P < 0.05$); while plants in the high and no fertilizer treatments did not differ significantly from one another. The density of active EFNs along the rachis length remained constant across fertilizer treatments ($F_{2,472} = 1.49$, $P = 0.23$). The number of EFNs with nectar significantly varied on leaves of different ages ($F_{2,474} = 155.03$, $P < 0.001$), with young leaves having more EFNs with nectar than both middle and old leaves, and middle leaves having significantly more than old leaves (Tukey-Kramer $P < 0.05$). Leaves of different ages responded differently to treatments (interaction; $F_{20,474} = 2.75$, $P = 0.03$), with young leaves responding more strongly to fertilizer treatments than old leaves.

Fertilizer amendment, leaf age, and maternal seed source all significantly affected the number of EFNs with visible nectar on plants grown in potting soil (Fig. 1.1D). Plants given high fertilizer had significantly more actively secreting EFNs per leaf than plants given medium fertilizer ($F_{1,1129} = 4.15$, $P = 0.04$). The density of active EFNs per rachis length was 29% greater in plants given high nutrient amendments than plants given medium fertilizer ($F_{1,1160} = 13.76$, $P < 0.001$). Young leaves had more EFNs containing nectar than both middle leaves and old leaves, and middle leaves had more than old leaves (Fig. 1.1D; $F_{2,1129} = 538.32$, $P < 0.001$, Tukey-Kramer $P < 0.05$). Leaves of different ages responded differently to treatment ($F_{2,1129} = 26.23$, $P < 0.001$).

Maternal lines significantly varied in the number of EFNs secreting nectar ($F_{24,1129} = 1.67$, $P = 0.02$), and in their response to treatment (interaction: $F_{24,1129} = 1.64$, $P = 0.03$). Of the maternal lines, 28% had more EFNs per leaf with nectar in high nutrients and 72% had no response to fertilizer treatments. There was also a significant interaction between leaf age and seed source ($F_{48,1129} = 1.94$, $P < 0.001$).

1.4.3 Cyanide production

For plants grown in native soil, the total amount of HCN released per leaf increased progressively with increasing fertilizer amendment (Fig. 1.2A; $F_{2,171} = 16.60$, $P < 0.001$; all treatment means were significantly different, Tukey-Kramer $P < 0.05$). On average, older leaves contained slightly (5%) more HCN per leaf than younger leaves (Fig. 1.2A; $F_{1,171} = 17.63$, $P < 0.001$). This increase in HCN release with age was highest in the

unfertilized plants, causing a marginally-significant interaction between fertilizer treatment and age ($F_{2,171} = 3.03$, $P = 0.05$).

Fertilizer treatments also increased the amount of HCN released per gram of leaf tissue damaged (Fig 1.2B; $F_{1,171} = 3.85$, $P = 0.02$). Averaging across leaves of different age, plants given high fertilizer released significantly more HCN per gram leaf than plants given medium and no fertilizer amendments (Tukey-Kramer $P < 0.05$), while plants in medium and no fertilizer treatments did not differ significantly. Young leaves released more HCN per gram tissue than older leaves (Fig 1.2B; $F_{1,171} = 32.36$, $P < 0.001$). A significant interaction between fertilizer treatment and leaf age (Fig. 1.2B; interaction $F_{1,171} = 4.26$, $P = 0.01$) is due to a disproportionately large age-related increase in leaf mass (Fig. 1C) relative to the increase in total leaf HCN (Fig. 1A) in the fertilized treatments. When young and old leaves were analyzed separately, comparison of treatment means within both age groups yielded the same outcome as the combined analysis: plants given high fertilizer produced more HCN per g than those given medium and no fertilizer, while medium and no fertilizer treatments did not differ significantly.

For plants grown in potting soil, fertilizer amendment did not affect the amount of HCN released per leaf (Fig. 1.2D; $F_{1,201} = 1.89$, $P = 0.17$). On average, young leaves released slightly, but significantly, more HCN than old leaves (Fig. 1.2D; $F_{1,201} = 6.85$, $P = 0.01$). Treatment and leaf age did not interact ($F_{1,201} = 1.24$, $P = 0.27$). Fertilizer treatments also did not affect the HCN per g leaf (Fig. 1.2E; $F_{1,201} = 0.02$, $P = 0.88$). Young leaves released more HCN per g than older leaves ($F_{1,201} = 149.93$, $P < 0.001$). This can be explained by a large increase in leaf mass (Fig. 1.2F) relative to the increase

in total HCN release per leaf as leaves age (Fig. 1.2D). In this trial, young and old leaves responded similarly to treatment ($F_{1,201} = 0.45$, $P = 0.50$).

Maternal seed source explained a significant amount of variation in HCN release per leaf ($F_{24,201} = 3.97$, $P < 0.001$) but did not explain variation in HCN release per gram leaf ($F_{24,201} = 1.37$, $P = 0.12$). This difference is likely an artifact of leaf size: the total amount of HCN released per leaf was dependent on leaf size, which was affected by seed source (see growth characteristics). Treatment did not interact with seed source ($F_{24,201} = 1.08$, $P = 0.37$) or leaf age ($F_{1,201} = 1.24$, $P = 0.27$) for HCN released per leaf and per gram leaf. The interaction between seed source and leaf age was not significant for HCN per leaf ($F_{24,201} = 1.18$, $P = 0.27$) but was for HCN per gram leaf ($F_{24,201} = 1.99$, $P = 0.005$).

1.4.4 Spine length

Although the spines of plants given high fertilizer were on average 20% longer than those of plants receiving medium fertilizer, the difference was not significant (Table 1.2, $F_{1,282} = 3.10$, $P = 0.08$). Maternal seed source had no effect on spine length ($F_{24,282} = 1.33$, $P = 0.14$), and there was no interaction between maternal line and treatment ($F_{24,282} = 0.84$, $P = 0.69$).

1.4.5 Soil properties

Based on soil particle size distribution, native soil was determined to be a sandy clay loam (Table 1.3). Native soil was 11 times denser than, and half as porous as, potting

soil (Table 1.4). Native soil contained very little AFDM. Prior to nutrient amendments, the orthophosphate concentration of native soil was about one sixth that of unamended potting soil. Both soils were low in NH_4-N, but native soil contained higher concentrations of nitrate than potting soil. Native soil contained about 7-fold higher NO_3-N concentrations than potting soil (Table 1.4). Native soil had about a 5-fold lower cation exchange capacity than the native soil (Table 1.4).

1.5 Discussion

We found that fertilizer additions on par with the average level of nutrient enhancement provided by ant colonies that nest under *Acacia constricta* increased acacia growth, EFN and active EFN numbers. Nutrient amendment intended to simulate the upper limit of nutrient amendment provided by ant nests resulted in increased HCN release from damaged leaves, but did not increase the numbers or activity of EFNs

The results of fertilizer treatment in this study depended upon the composition of the soil in which acacias grew. We assume that the responses of plants grown in their native soil are more representative of acacias in their natural environment than those of plants grown in potting soil, therefore we emphasize the importance of the native soil results. However, differences between the two trials may provide insight into mechanism.

Nutrient amendment increased damage-induced cyanide release by *A. consticta* grown in native soil (Fig. 1.2B). This is consistent with other findings that elevated

nitrogen increases cyanogenesis (Dement and Mooney 1974; Forslund and Jonsson 1997; Burns et al. 2002). The effect of nutrient amendment on cyanogenesis was particularly strong for young leaves (Fig. 1.2B), which are typically more vulnerable to herbivory under natural conditions. Cyanogenesis deters feeding by mammals and many generalist insect herbivores (reviewed by Gleadow and Woodrow 2002). However, in some cases cyanide production can actually serve as a phagostimulant to insect herbivores (e.g., Mowat and Clawson 1996). The results suggest that ant nests particularly rich in soil nutrients may enhance chemical defense in *A. constricta*.

There were significant differences between young and old leaves in average HCN production per leaf in both experimental trials (Fig. 1A and 1D), but the magnitude of these differences was small, suggesting that plants provision leaves with HCN precursors early in leaf development and that the total HCN-releasing potential of a leaf does not change radically as leaves age. Per g of leaf tissue, fully expanded leaves had lower potential for HCN release than young leaves (Fig. 1B and 1E). This is consistent with the hypothesis that leaf expansion dilutes cyanogenesis (Hayden and Parker 2002; Gleadow and Woodrow 1999). However, Gleadow and Woodrow (1999) found that a decrease in cyanogenesis in older leaves is due to both dilution with growth as well as reduction in the proportion of leaf nitrogen allocated to cyanogenesis.

While plants grown in native soil responded to nutrient addition by producing more HCN, there was no evidence that nutrients limited HCN production in plants grown in potting soil (Fig 1.2). A further difference between the two trials was the quantity of HCN released. Plants in potting soil released on average 41% more μg HCN more per

gram leaf than plants grown in native soil. HCN is a nitrogen-based compound and has been shown to increase in response to increased nitrogen (Dement and Mooney 1974; Forslund and Jonsson 1997; Burns et al. 2002). At a given level of amendment, plants growing in potting soil likely had greater access to nutrients than those in native soil. Two lines of reasoning support this. First, fertilizer treatments were added per volume soil. Though potting soil had a lower initial nitrate concentration than native soil, it had a much lower bulk density than native soil (Table 1.4), so that the potting soil was given more fertilizer per gram soil than native soil. Second, because the potting soil had a five-fold greater cation exchange capacity than native soil (Table 1.4), it could retain more ammonium ions, likely increasing the availability of nutrients to plants.

Modest levels of nutrient amendment initially had a positive effect on both the number of EFNs and actively secreting EFNs per leaf on *Acacia constricta*. Since EFNs are arranged linearly along the rachis, the most parsimonious mechanism for an increase in EFN number with resources might be that EFN number necessarily increases as the leaf lengthens. Our results from plants grown in native soil support this reasoning. However, the density of EFNs per leaf on plants grown in potting soil increased when given higher nutrients, suggesting EFN number is controlled independent of leaf expansion.

If EFN number is controlled independently of leaf growth, then an increase in EFN numbers with fertilization may represent a change in resource allocation to EFNs. Low numbers of EFNs in the no fertilizer treatment may reveal a cost of EFN construction and maintenance (Rutter and Rausher 2004), and may suggest that EFNs and

nectar sectretion are both carbon and nutrient limited. The lack of further increases in EFN number between medium and high fertilizer treatments combined with the increase in HCN concentration with higher nutrient amendment may indicate a shift in allocation from more carbon-based structures (EFNs) to nitrogen-based defense chemicals as the carbon to nitrogen ratio within a plant decreases.

Ant visitation to plants is positively related to EFN secretion (Heil et al 2001; Linsenmair et al. 2001; Ness 2003), and the experimental addition of nectaries to *A. constricta* plants increases the probability that ants will nest underneath plants (Wagner and Nicklen, in prep). For nutrient-limited plants, the results of the present study suggest that modest increases in soil fertility by ant nests could fuel a feedback loop whereby ant-added nutrients increase ant attractants (EFNs), which in turn encourage more ant visitation and nesting. We do not, however, know the effect of fertilization on the volume of nectar secreted per EFN.

The observed effect of maternal seed source on numbers of EFNs per leaf could mean that EFN production is affected by maternal environment, maternal genetic material, chromosomal genetic material, or a combination. Energy and nutrients provisioned within the maternal tissues in which the zygote develops can influence the phenotype of the plants after germination (Parrish and Bazzaz 1985; Roach and Wulff 1987). Although maternal environmental effects are often limited to the early growth of the plant (Miao et al. 1991, Wulff and Bazzaz 1992; Schmid and Dolt 1994), they can affect offspring defense levels (Agrawal 1999). EFN production might also be influenced by maternal genetic material found in mitochondria, chloroplasts, or plastids

(Roach and Wulff 1987), or by genetic material on chromosomes. Few studies have investigated the heredity of EFNs, but in *Gossypium*, two pairs of recessive genes have been found to determine the presence or absence of nectaries (Meyer and Meyer 1961, Rhyne 1965).

In contrast, we did not find an influence of maternal seed source on cyanogenesis or spine length. Using a similar approach to ours, Schappert and Shore (1995) found statistically significant effects of maternal seed source on cyanogenesis, although their ability to detect a maternal effect depended somewhat on plant age. To our knowledge, no other studies have examined whether variation in spine length is genetically determined.

The standard approach to studying how the aggressive behavior of visiting ants affects herbivory is to experimentally exclude ants from plants using a sticky barrier. If ants commonly "feed" plants, whether via soil nutrients or through nesting chambers, these fertilization effects could confound the interpretation of ant-exclusion experiments. Exclusion experiments remove the benefits of ants attacking plant predators, but may also remove unseen benefits of ant-increased nutrients, such as increased HCN release. If this is the case, exclusion experiments may overestimate the benefit of aggressive ant behavior. It is also possible that the positive effects of ant-added nutrients linger after ants have been excluded. In this case, the benefits of ant presence may be underestimated. Our results suggest that a more careful examination of the mechanism driving the results of future ant exclusion experiments is warranted.

1.6 References

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Table 1.1: Field and experimental soil nutrient levels. Phosphorus, nitrate, and ammonium levels found in soil beneath *Acacia constricta* growing in Arizona (Wagner 1997) and the amount of nutrients added to experimental plants. Medium fertilizer treatment approximates the nutrients found beneath an average *F. perpilosa* nest, while the high treatment approximates nests with the highest nutrient levels found in the field. The level of osmocote added was based on native soil mass (~990g/32oz cup).

Treatment	Nutrient	Field nutrient level (μg/ g soil)	Nutrients added (μg/ g native soil)	Osmocote (g/application)
Medium	P	54	54	0.884
	NO_3^-	40	51	
	NH_4^+	13	72	
High	P	123	123	2.013
	NO_3^-	155	117	
	NH_4^+	65	165	

Table 1.2: Mean spine length and growth characteristics. Mean (± 1 SE) values for spine length, total number of leaves per plant, dry shoot, root, and root nodule mass for plants in potting soil, and plant height and leaf rachis length for plants in both native and potting soil.

	Native soil			Potting soil	
Plt trait	No fertilizer	Medium	High	Medium	High
Spine(mm)	---	---	---	2.02 ± 0.10^{a}	2.43 ± 0.15^{a}
Height(cm)	38.2 ± 3.7^{a}	59.9 ± 4.5^{b}	54.3 ± 3.4^{b}	40.1 ± 1.1^{a}	42.3 ± 1.3^{a}
Rachis(cm)	2.8 ± 0.1^{a}	3.7 ± 0.1^{b}	3.6 ± 0.1^{b}	3.0 ± 0.1^{a}	3.1 ± 0.1^{a}
#Lvs/plt	---	---	---	127 ± 7^{a}	140 ± 7^{a}
Dry shoot(g)	---	---	---	8.2 ± 0.2^{a}	9.5 ± 0.3^{b}
Dry root(g)	---	---	---	4.8 ± 0.3^{a}	5.9 ± 0.3^{b}
Dry nodule(g)	---	---	---	0.34 ± 0.04^{a}	0.39 ± 0.04^{a}

Within a soil type, means annotated with different letters are significantly different (Tukey-Kramer, $P<0.05$).

Table 1.3: Soil particle distribution for native soil.

Particle size	Percent composition
Gravel (% of sample)	16.31 ± 0.40
Sand (% of soil)	50.18 ± 0
Silt (% of soil)	20.77 ± 0.22
Clay (% of soil)	29.05 ± 0.22

Table 1.4: Physical and chemical properties of native and potting soils (mean ± 1 SE). *P*-values are from t-tests with n=6.

Measurement	Native soil	Potting soil	*P*-value
Bulk density (g/cm^3)*	1.39 ± 0.01	0.12 ± 0.002	< 0.001
Porosity (%)*	47.56 ± 0.49	95.47 ± 0.39	< 0.001
AFDM (mg/g dry soil) †	15.59 ± 0.71	614.22 ± 8.66	< 0.001
Initial P (µg/g soil)	14.48 ± 0.27	95.40 ± 2.40	< 0.001
Initial NH_4-N (µg/g soil)	0.56 ± 0.15	1.79 ± 0.21	0.009
Initial NO_3-N (µg/g soil)	10.32 ± 0.43	1.43 ± 0.21	< 0.001
pH	6.56 ± 0.01	5.64 ± 0.01	< 0.001
CEC ($cmol_c$/kg soil)	9.71 ± 0.17	47.19 ± 4.50	< 0.001

*Both porosity and soil particle distribution are based on a particle density of 2.65 g/cm^3.

†AFDM is ash-free dry mass.

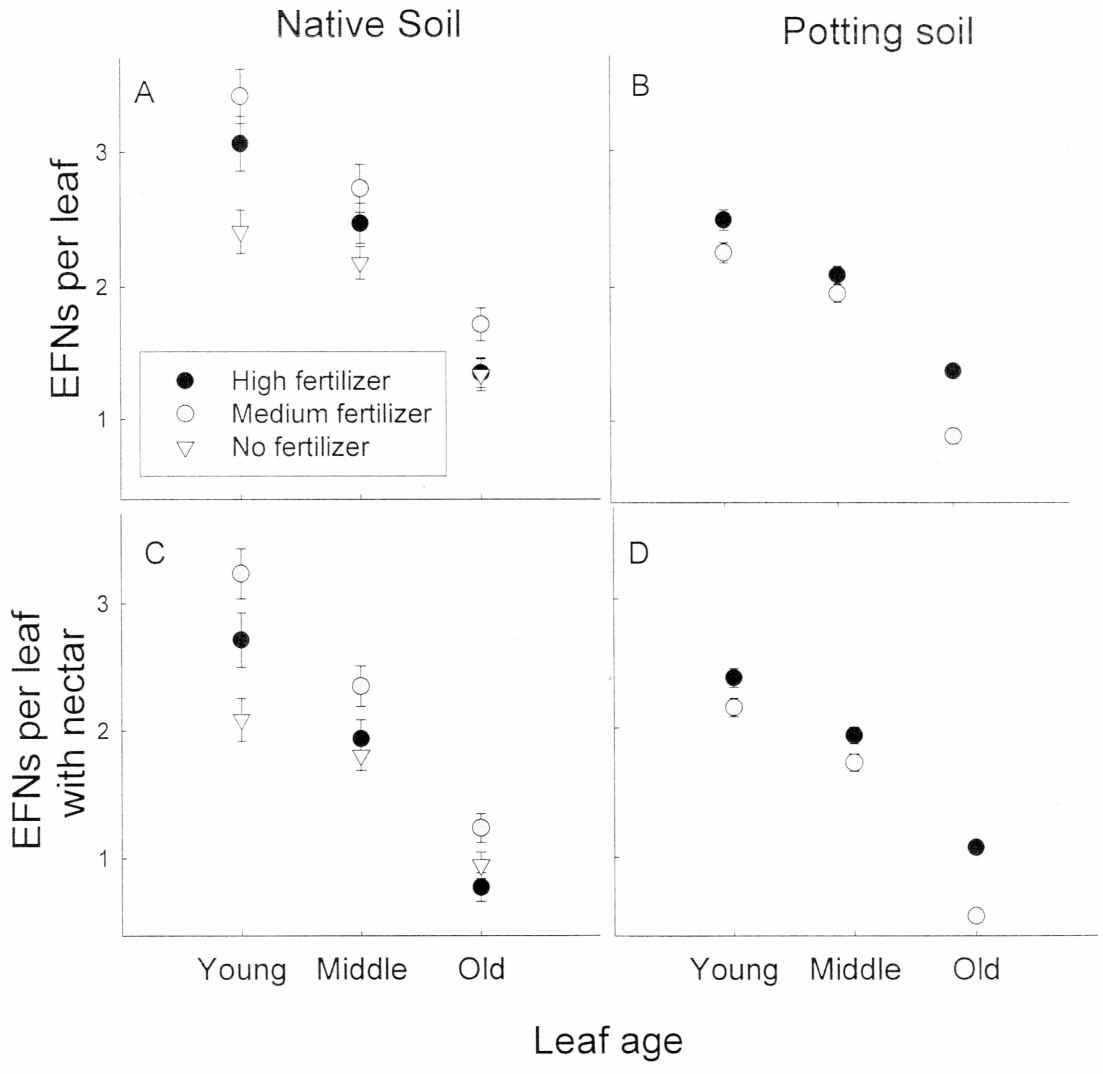

Figure 1.1: Effects of nutrient amendment on EFN number and secretion for leaves of differing age. Mean (± 1 SE) number of EFNs and actively secreting EFNs per leaf for plants grown in native soil (A,C) and potting soil (B,D) in no fertilizer, medium, and high fertilizer.

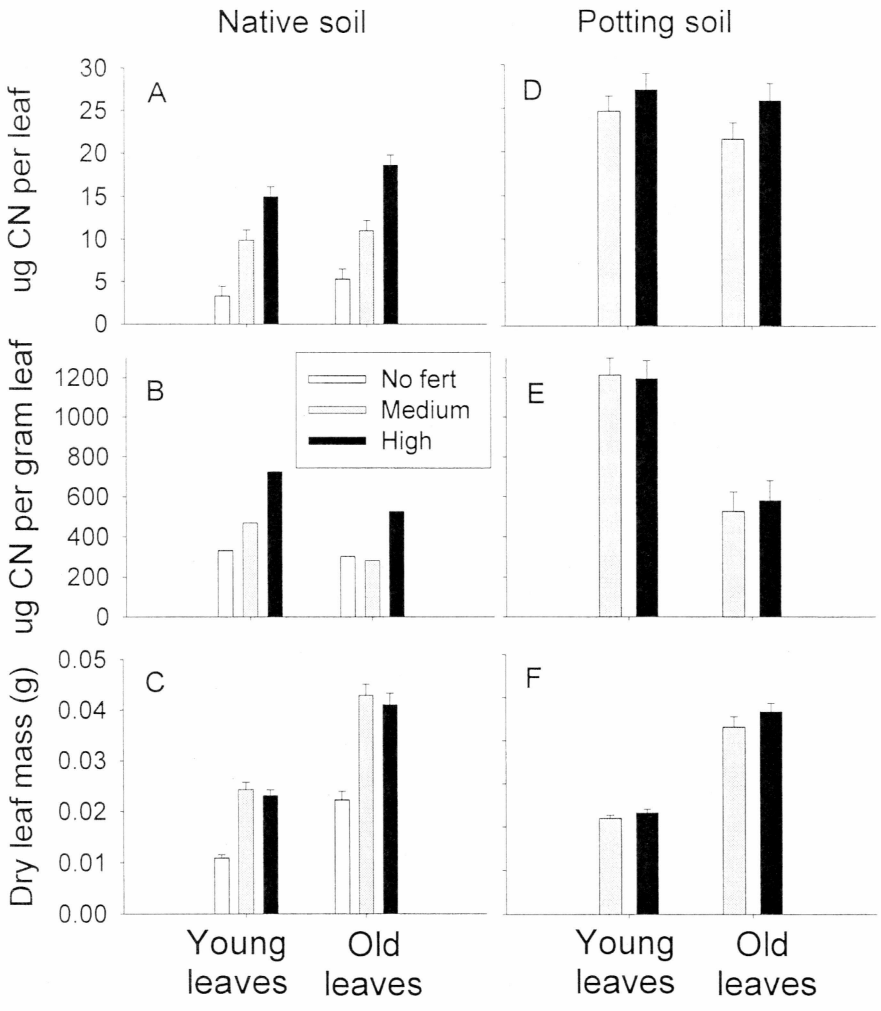

Figure 1.2: Effects of nutrient amendment on HCN production on young and old leaves. Mean (± 1 SE) micrograms of HCN released per gram leaf and per leaf from plants grown in native soil (A, B) and potting soil (D, E) and given no fertilizer, medium, and high fertilizer. HCN per leaf and per g leaf are least squared means. Mean dry leaf mass is shown for plants in native (C) and potting soil (F).

Chapter 2 Conflict resolution in an ant-plant interaction: *Acacia constricta* traits reduce ant costs to reproduction[2]

2.1 Abstract

Many plant species attract ants onto their foliage with food rewards or nesting space. However, ants can interfere with plant reproduction when they visit flowers. This study tests whether *Acacia constricta* separates visiting ant species temporally or spatially from newly opened inflorescences and pollinators. The diurnal activity patterns of ants and *A. constricta* pollinators peaked at different times of day, and the activity of pollinators followed the daily dehiscence of *A. constricta* inflorescences. In addition to being largely temporally separated, ants rarely visited open inflorescences. A floral ant repellent contributes to the spatial separation of ants and inflorescences. In a field experiment, ants of four species were given equal access to inflorescences in different developmental stages. On average, the frequency with which ants made initial, antennal contact with the floral stages did not differ, but ants significantly avoided secondary contact with newly opened inflorescences relative to buds and old inflorescences, and old inflorescences relative to buds. Ants also avoided contact with pollen alone, indicating that pollen is at least one source of the repellent. The results suggest *A. constricta* has effectively resolved the potential conflict between visiting ants and plant reproduction.

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[2] Nicklen EF, Wagner D (2006) Conflict resolution in an ant-plant interaction: *Acacia constricta* traits reduce ant costs to reproduction. Oecologia 148: 81-87

2.2 Introduction

All interspecific interactions involve conflicts of interest between the species involved. The resolution of the conflict defines whether the relationship is mutualistic or antagonistic (Bronstein 2001). Ant-associated plants are in such a conflict with their visiting ants. Ants can increase plant fitness by defending against herbivores, pruning encroaching vegetation, reducing fungal and bacterial growth, and increasing soil nutrients (Janzen 1966; Bentley 1977; Beattie 1985; Beattie et al. 1986; Madden and Young 1992; Davidson and McKey 1993; Wagner 1997; Letourneau 1998; Stapley 1998; Sager et al. 2000; Fischer et al. 2003). Yet, ants can also have negative impacts on components of plant fitness by both reducing pollinator visitation and rendering pollen inviable. Ants can discourage pollinator visitation indirectly, by robbing nectar or simply being on the plant, and directly, by chasing or attacking pollinators (McDade and Kinsman 1980; Normant 1988; Buys 1990; Galen 1999). Furthermore, most ant species secrete an antibiotic substance from the metapleural gland onto the integument that reduces pollen viability (Beattie et al. 1984; 1985; 1986). Plant species in which ant visitation is common possess a variety of mechanisms, often morphological or chemical, that reduce the cost of ants to plant reproduction (Feinsinger and Swarm 1978; Guerrant and Fiedler 1981; Harley 1991; Federle et al 1997; Willmer and Stone 1997; Galen 1999; Ghazoul 2001; Raine et al. 2002; Wagner and Kay 2002).

Much of our understanding of ant-plant-pollinator interactions comes from the genus *Acacia* (subfamily: Mimosideae, family: Fabaceae). Acacias attract ants onto the

foliage with extrafloral nectaries (EFNs), nectar-secreting glands found on leaves. In some species swollen thorns (nesting space) and protein-rich Beltian bodies further attract ants. Many acacias require out-crossing by pollen vectors (Kenrick and Knox 1989; Kenrick 2003), setting up a potential conflict between the ants and pollen vectors. Additionally, *Acacia* species are pollinator generalists (Bernhardt 1989); their stamens and stigmas are exposed to anything that lands or crawls over the flowers, making acacia pollen particularly susceptible to the metapleural secretions of ants. Acacias, as well as other plants with exposed stamens, have likely evolved non-morphological mechanisms to resolve the potential conflict among ants, flower visitors, and pollen.

In this study we investigate mechanisms that may reduce conflicts between *Acacia constricta* and visiting ants, which can have harmful effects on plant reproduction. *Acacia constricta* plants associated with the ant, *Formica perpilosa*, set more seeds than plants not associated with ants (Wagner 1997). However, *F. perpilosa* ants reduce pollen viability when they contact flowers (Wagner 2000). This study tests the hypothesis that *A. constricta* segregates four common ant species from flowers and pollinators by presenting pollen at periods of low ant activity and by producing a floral ant repellent. We also test the hypothesis that pollen is the source of the ant repellent.

2.3 Materials and methods

2.3.1 Study system and species

The study was conducted 5km northeast of Portal, Arizona, at an intersection of the Chihuahuan and Sonoran deserts (31°54'01" N, 109°05'26 W). Vegetation at the site was dominated by *Prosopis juliflora* (mesquite) and *Acacia constricta*. Tests were conducted from mid July to mid September 2004.

Acacia constricta is a deciduous shrub that produces leaves and inflorescences following heavy rains that typically occur in July and August. Flowering typically ceases in late September or October, and seeds ripen by late October or November. Inflorescences are yellow, spherical, largely self-incompatible (Wagner 2000), about 10mm in diameter, and contain 25 to 80 flowers each. Flowers within an inflorescence open virtually simultaneously and produce no detectable nectar. Pollen is presented in polyads, with 16 grains per polyad. Leaves bear extrafloral nectaries (EFNs) along the rachis.

Acacia constricta is associated with the ant, *Formica perpilosa*. *Formica perpilosa* feeds on nectar secreted by *A. constricta's* EFNs as well as caterpillars and homopterans. At the study site, *F. perpilosa* colonies form permanent nests under *Prosopis juliflora*. Colonies expand after the summer rains, forming satellite nests under *A. constricta*. Although there is no evidence that ants reduce herbivory, plants with basal ant nests have significantly higher soil nutrients at their base and produce about twice as many seeds as plants without basal nests (Wagner 1997). Bioassays have shown that

Formica perpilosa significantly reduces pollen viability of *A. constricta* upon contact (Wagner 2000). Three other ant species, *Myrmecocystus mimicus, Dorymyrmex* sp. *(smithi* complex), *and Forelius pruinosus*, commonly visit *A. constricta.* All of these species have metapleural glands and likely reduce pollen viability as well (Beattie et al. 1984, 1985).

An important herbivore on *A. constricta* at the study site is the lycaenid caterpillar *Hemiargus isola.* Eggs are laid singly on flower buds. Third and fourth (final) instar caterpillars shift from buds to open inflorescences, where they consume, and efficiently digest, pollen (Wagner and Martínez del Rio 1997). Caterpillars are tended by at least four species of ants, to which they secrete food rewards upon demand. Ant tending increases *H. isola* survivorship and growth rates (Wagner 1993; Wagner 1995; Wagner and Kurina 1997).

2.3.2 Temporal separation

2.3.2.1 Dehiscence

To test the diurnal pattern of dehiscence, we sampled the pollen to anther ratio on *A. constricta* inflorescences. We obtained the pollen to anther ratio of an inflorescence by lightly dabbing the inflorescence on clear adhesive tape, placing the tape on a microscope slide, and counting the polyads and anthers (Stone et al. 1998). Before dehiscence only young anthers are removed on the tape. The pollen to anther ratio rises as pollen is released and decreases as pollinators remove pollen. We sampled 6 inflorescences (3

unbagged and 3 bagged) on each of 6 plants approximately every two hours from 05:30 to 17:30 on 19 August 2004. From 06:00 to 12:00 on 22 September and from 13:00 to 18:00 on 21 September 2004, we sampled 3-5 unbagged inflorescences hourly on each of 4 different plants. The difference in ratio between the bagged and unbagged flowers presumably reflects pollen removal by insects.

2.3.2.2 Ant-pollinator observation

We conducted two studies to determine the overlap in temporal activity patterns of ants and putative pollinators. The first involved direct observation of ants and flower visitors on *A. constricta*. For 5 minutes we watched *A. constricta* plants with at least 2 newly-opened inflorescences and recorded the number and species of each flower visitor. Immediately after the 5-minute observation period, we counted the number and species of ants on the entire plant. Ants were counted on the foliage nearest the observer while walking around the plant at a constant rate; a census of a typically-sized shrub lasted about 1 minute. Observations were conducted continuously, moving from plant to plant, from 06:00 to 18:00 (PDT). Over the course of six days (3 mornings and 3 afternoons), 170 observations were conducted on 84 flowering plants. Air temperature during the study was recorded with a Hobo data logger.

2.3.2.3 Pan traps

In addition to observations, we monitored the diurnal activity patterns of putative pollinators and ants by trapping insects throughout the day in bowls filled with soapy

water (pan traps). Although pan traps are typically used for catching flying insects and estimating pollinator abundance (LeBuhn 2003 et al.), ants also commonly appeared in our traps as well. The number of ants and bees collected during a time interval presumably reflected the activity of ants and bees at that time. Fifteen bowls, two thirds of which were white and one third yellow, were set approximately 3 m apart on open ground along a single transect near large flowering *A. constricta*. Contents of bowls were collected hourly from 06:00 to 18:00 for 3 mornings and 3 afternoons. Bees were identified to genus and ants to species. Bees caught in the bowls were examined under a microscope and scored for the presence or absence of *A. constricta* pollen.

2.3.3 Spatial separation

In order to determine if, and how often, ants visit inflorescences, we quantified the numbers of ants visiting different plant tissues. While counting the number of ants per plant as described above, we also tallied the number of ants on branches, leaves, new inflorescences, old inflorescences, and buds. New inflorescences had opened within 24 hours and were bright yellow. Old inflorescences were 2-3 days old, dark yellow to brown, and losing flowers. To investigate finer-scale patterns of ant visitation, we focused more intensively on a set of 12 plants, chosen because they had relatively high ant visitation. Three times during the morning (07:00 – 10:00, PDT) for three days, we counted the number of ants on stems, leaves, buds, new inflorescences and old inflorescences of each plant. For the latter data set, we compared the average number of

ants per plant visiting the three floral stages using ANOVA, with plant as a blocking factor.

2.3.4 Floral ant-repellent

We tested three predictions about floral repellence in *A. constricta*. 1) Newly-dehisced inflorescences (hereafter "new inflorescences") repel ants. 2) The repellent is detected before contact rather than upon contact. 3) The repellent is effective against a wide range of ant species in the community.

To test these predictions, we measured the rate at which ants of four species contacted buds, new inflorescences, and old inflorescences. We confirmed the presence of pollen on new inflorescences before including them in the experiment. We placed one of each of the three floral stages in a semi-circle approximately 4 cm from a central source of sugar water, used to attract ants to the area of the experiment. For *Formica perpilosa*, who make their permanent nests under *Prosopis juliflora*, we put the floral stages and sugar water on 10 x 15 cm platforms mounted in *P. juliflora* branches (N = 14 colonies). For *M. mimicus*, *Dorymyrmex* sp., and *F. pruinosus*, who have entrances on open ground, the floral stages were placed around the nest entrance (N = 20 colonies for each species). We situated the sugar water between the nest entrance and the floral stages. For *F. perpilosa* colonies, we placed sugar water next to the main entrance onto the tree platform (the branch to which the platform was attached), such that the sugar water was between the inflorescences and the main entrance. We conducted these experiments between 07:30 and 11:00 for each ant species.

We recorded the number and type of ant contacts to each floral stage during a 10-minute observation period. After touching an inflorescence with the antennae, ants either moved away or extended contact by moving onto the inflorescences. We categorized ant contacts as exploratory, involving contact with antennae only, or protracted, involving contact with one or more legs as well.

If new inflorescences emit a repellent that ants detect before contact, then ants should make fewer mean total contacts (explorative + protracted) to new inflorescences than to buds or old inflorescences. If the repellent is detected upon contact, then the mean proportion of all contacts that are protracted should be lower for new inflorescences than for buds or old inflorescences. We examined the effect of floral stage on total number of contacts and proportion of contacts that were protracted using separate linear mixed model ANOVAs (PROC MIXED, SAS institute 2004). Floral stage, ant species, and the interaction between floral stage and species were treated as fixed effects and ant colony by species was included as a random factor. If floral stages explained a significant amount of variation in the models, we conducted pairwise comparisons of means using Tukey-Kramer tests.

2.3.5 Pollen repellent

To test whether pollen itself is repellent to ants, we placed agar on opposing ends of a microscope slide and tapped newly-opened inflorescences above the agar on one side so that pollen dusted the agar. If anthers, as well as pollen, stuck to the agar, the slide was not used. We placed slides approximately 5 cm from nest entrances. If ant activity was

low, we placed sugar water baits equidistant from the two ends of the microscope slide. We counted the number of times an ant crossed the control and pollen agar within a 5-minute period. Slides were not reused. We tested 14 colonies of *F. perpilosa* and 20 colonies each *M. mimicus*, *Dorymyrmex* sp., and *F. pruinosus* (one trial per colony). We compared the number of contacts to the control and pollen agar using a linear mixed model (PROC MIXED) with fixed effects of treatment, species, and their interaction, and a random factor of colony by species (SAS institute 2004).

For all analyses, data were tested for equality of variances and model residuals were examined for normality. Data were log transformed when necessary to meet parametric assumptions.

2.4 Results

2.4.1 Temporal separation

2.4.1.1 Dehiscence

Pollen dehiscence began around 08:00 and peaked between 11:00 and 12:00 on 19 August and between 10:00 and 11:00 on 21 and 22 September (Fig. 2.1A). After 12:00, the pollen to anther ratio of unbagged inflorescences declined more rapidly than bagged inflorescences, likely due to pollen removal by bees.

2.4.1.2 Ant-pollinator observation

Bee and ant activity peaked at different times of day (Fig. 2.1B). Bee activity on *A. constricta* plants followed a similar pattern to dehiscence, beginning between 08:00 and 09:00, increasing to its peak between 11:00 and 12:00, and ending between 15:00 and 16:00 (Fig. 2.1B). On the other hand, ants were active mainly in the mornings and evenings, with peak activity between 08:00 and 09:00 and between 16:00 and 17:00 (Fig. 2.1B). A period of overlap of bee and ant activity occurred between 08:00 and 10:00 (Fig. 2.1B). The time of overlap between ant activity and pollen availability extended from 06:00 to 15:00, with the most extensive overlap between 07:00 and 10:00 (Fig. 2.1B).

Over the course of observations, we counted 66 flower visitors, (73% of which were bees). Most visiting bees carried visible pollen loads on arrival and crawled in circles over the inflorescence collecting pollen. The only other visitor that appeared to carry pollen in its hairs was a beetle (*Acmaeodera* sp.) (16.7% of visitors). These beetles consumed flowers, likely making them poor pollinators. Visiting bee taxa included *Dialictus* spp. (Halictidae) (50% of visiting bees), *Exomalopsis* spp. (Anthophorinae) (12.5%), an unknown genus (Anth

2.4.1.3 Pan traps

Bees and ants collected in pan traps followed a pattern similar to those observed on plants. Bees were trapped from around 08:00 until 15:00, with peak abundance between 10:00 and 11:00 (Fig. 2.1C). Ants were most abundant in pans traps from 06:00 to 10:00 and from 15:00 to after 18:00 (Fig. 2.1C). Again, an overlap in ant and pollinator diurnal activity occurred between 08:00 and 10:00 (Fig. 2.1C). The contents of pan traps included 68 bees representing 9 species and 91 ants in 4 species (*95% Dorymyrmex* sp). Eight of the bee species collected had a least one representative carrying *Acacia constricta* pollen, suggesting these species at least occasionally visit *A. constricta*.

Temperatures during observations and pan-trapping ranged from 16° to 52°C. The average max

plants, we observed ants on 1% (± 0.7 SE) of all buds, 3.5% of new inflorescences (± 1.7) and 0.7% (± 0.1) of old inflorescences.

More intensive sampling of individual plants indicated that, unless a tended caterpillar was present, ant visitation to inflorescences fell after flower buds opened. These plants were visited by an average of 6.1 ants each and contained at least 40 inflorescences in each developmental stage. Averaging across plants, 11.9% (±1.6 SE) of ant visits were observed on inflorescences: 4.1% (±0.6) on buds, 5.4% (± 1.3) on new inflorescences, and 2.4% (± 0.5) on old inflorescences. Of the ants visiting new inflorescences, 65% were tending *H. isola* caterpillars, 20% were prying into flowers, and 15% were walking over inflorescences. Overall, ants were slightly more likely to be observed on new flowers than buds or old inflorescences, but there was no statistically significant difference among floral stages (Fig 2; log-transformed data, $F_{2,22} = 3.1$, P

Fig. 2.3A suggests that *F. pruinosus* responded as predicted and avoided new inflorescences more than buds and old inflorescences. *Dorymyrmex* sp. and *F. perpilosa* contacted new inflorescences as much or more than other stages and *M. mimicus* appeared to favor contact with buds over new and old inflorescences (Fig. 2.3A)

The proportion of ant contacts that was protracted varied significantly among floral stages ($F_{2,137} = 34.9$, $P < 0.001$). Ants strongly avoided protracted contact with new inflorescences relative to buds and old inflorescences (Tukey-Kramer $P < 0.001$), and avoided old inflorescences slightly, but significantly, more than buds (Tukey-Kramer $P < 0.05$). This result suggests there is a floral repellent and it is detected upon contact. Ant species varied significantly in their response to floral stage (Fig. 2.3B; $F_{3,70} = 3.9$, $P = 0.013$). Although there was a significant interaction between ant species and floral stage ($F_{6,137}$=2.8, $P = 0.013$), the rank order of mean responses to the three floral stages was identical for all ant species.

2.4.4 Pollen repellent

Ants significantly avoided agar dusted with pollen relative to controls (Fig. 2.4; $F_{1,70} = 23.8$, $P < 0.001$). The number of contacts to agar did not differ among species (Fig. 2.4; $F_{3,70} = 0.8$, $P < 0.5$) and there was no significant interaction between ant species and treatment (Fig. 2.4; $F_{3,70} = 0.8$, $P = 0.5$).

2.5 Discussion

In this study, we established that *Acacia constricta* separates ants from new inflorescences and pollinators in a least two ways. First, *A. constricta* inflorescences dehisce when visiting ants are least active, temporally separating ants from pollen and pollinators. *Acacia zanzibarica* also dehisces when ants are least active (Willmer and Stone 1997). However, temporal separation of ants from new inflorescences and pollinators is not found in all *Acacia* species. For instance, *A. hindsii* shows less temporal separation (Raine et al. 2002), and ants and pollinators are active at the same time of day on *A. collinsii* (Ghazoul 2001). Second, *Acacia constricta* has a floral ant repellent, which may be a trait common to *Acacia* species. Including this study, six *Acacia* species, comprising species with ant association (*A. collinsii, A. constricta, A. hindsii, A. zanzibarica*), species without ant association *(A. angustissima, A. macracantha)*, species from Africa (*A. zanzibarica)*, and species from the neotropics, have been tested and all have displayed a floral ant repellent (Willmer and Stone 1997; Ghazoul 2001; Raine et al. 2002).

Most ant species in this study detected *A. constricta* floral repellent upon contact rather than before contact. Our findings suggest that the repellent is not particularly volatile. A highly volatile substance would likely have affected the overall frequency with which ants contacted inflorescences, rather than the frequency of protracted contacts alone. This appears consistent with the response of ants to floral repellents in other plant species where ants spend less time on newly opened flowers (Willmer and Stone 1997) and on petri-dishes wiped with newly opened flowers (Ghazoul 2001). Similarly, Raine

et al. (2002) found ants to either "pass through" areas of stem wiped with newly opened flowers or to halt at the wiped boundary, suggesting ants must closely approach the area of repellence to detect it.

In particular, we found that ants avoided contact with *A. constricta* pollen (Fig. 2.4). This finding is the first to support the hypothesis that pollen is a component, if not the sole source, of the floral ant repellent. It is also possible that anther glands are repellent. Anther glands are globular structures attached to the top of an anther by a stalk and are found in many *Acacia spp.* (Kenrick 2003). These glands may play a role in producing floral scents and function as pseudo-pollen or a true food reward to attract pollinators when stigmas are receptive (Stone et al. 2003). In our tests, along with the pollen, a few anther glands were dusted onto the agar. Since anther glands are only slightly larger than pollen and fall from anthers like pollen, it was not feasible to exclude them from the agar. Thus, anther glands cannot be eliminated as a potential repellent source.

Acacia constricta pollen repelled all four visiting ant species, representing two subfamilies, suggesting the repellent is effective on a broad range of ant species. Since multiple ant species often visit plants (Koptur 1992, McKey and Davidson 1993), the ability of a plant to repel a range of ant species from flowers is an important adaptation. Yet, the repellent appears to be specific enough to ants that their hymenopteran relatives, the bees, are not also repelled.

The majority of ants observed on new inflorescences were tending caterpillars of the lycaenid butterfly species *H. isola*. Because *H. isola* caterpillars consume pollen,

they typically occupy new inflorescences most repellent to ants. Although it is clear that the attractiveness of the lycaenid caterpillars often overrides the repellency of pollen, it is possible that the floral repellent affects such factors as the number of ants tending caterpillars or the constancy of attendance. Ant tending increases *H. isola* larval growth rates and survivorship (Wagner 1993; Wagner and Kurina 1997), so if the floral repellent discourages ants from tending, it could also act to reduce florivory.

In field surveys and experiments, ants avoided open flowers, both new and old, relative to buds. Avoidance of new inflorescences is consistent with our experimental evidence that pollen is repellent. Residual pollen may account for ant avoidance of old inflorescences. Ants might avoid open inflorescences for other reasons as well, such as poor footing. Clusters of protruding stamens found on open inflorescences may be more difficult to walk over than the more compact buds.

Prior to this study, the effect of ants on male and female function in *A. constricta* appeared to conflict. Positive effects of *Formica perpilosa* on seed set (Wagner 1997) appeared to be accompanied by reduced pollen viability when ants contacted flowers (Wagner 2000). Here we show *A. constricta* reduces potential costs to male function using mechanisms that limit contact between ants and flowers, while still maintaining the benefits of ants. Investigation of the conflicts inherent in interspecific interactions and how those conflicts are resolved contributes to our understanding of how mutualisms, such as those between ants and plants, persist.

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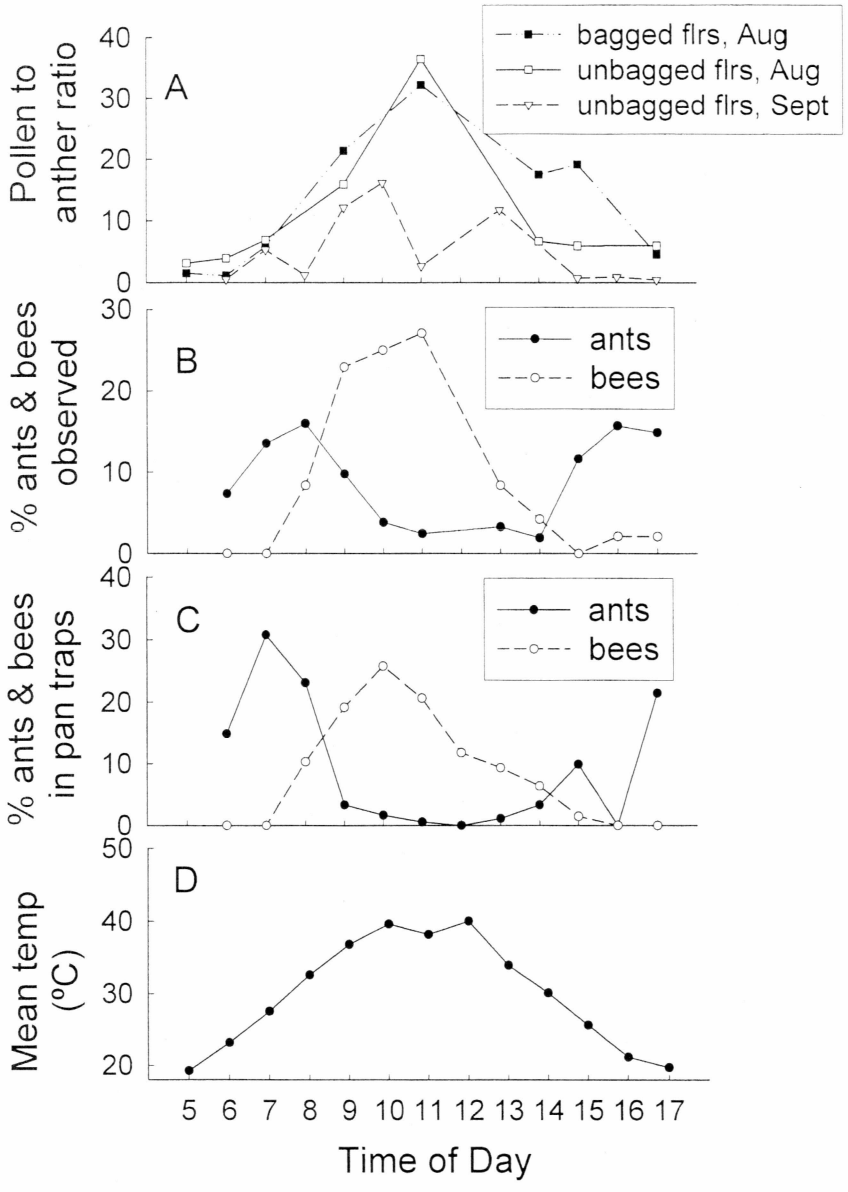

Figure 2.1: Diurnal trends. A. Daily pattern of dehiscence for bagged and unbagged inflorescences on 19 Aug and 21- 22 Sept. B. Percentage of ants (N=370) and bees (N=48) observed on *A. constricta* at each time interval. C. Percentage of ants (N=91) and bees (N=68) caught in pan traps at hourly intervals. D. Average temperatures during ant-pollinator observations and pan-trapping (15, 16, 18, 22, 21, 23 Sept 2004). Time of day represents hourly intervals.

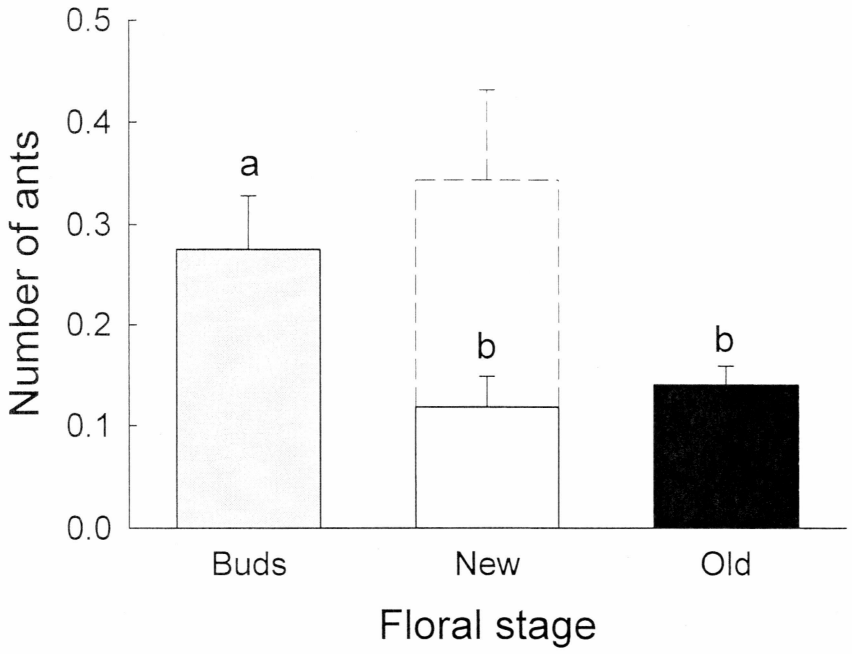

Figure 2.2: Number of ants observed on buds, new, and old inflorescences. Mean (± 1 SE) number of ants visiting *A. constricta* flowers in three developmental stages. Bars drawn in solid lines represent the number of ants visiting inflorescences, excluding those tending lycaenid caterpillars. Bars annotated with different lower case letters are significantly different using Tukey-Kramer HSD ($P < 0.05$). The dashed bar is the overall mean number of ant tending new inflorescences, and includes those tending caterpillars.

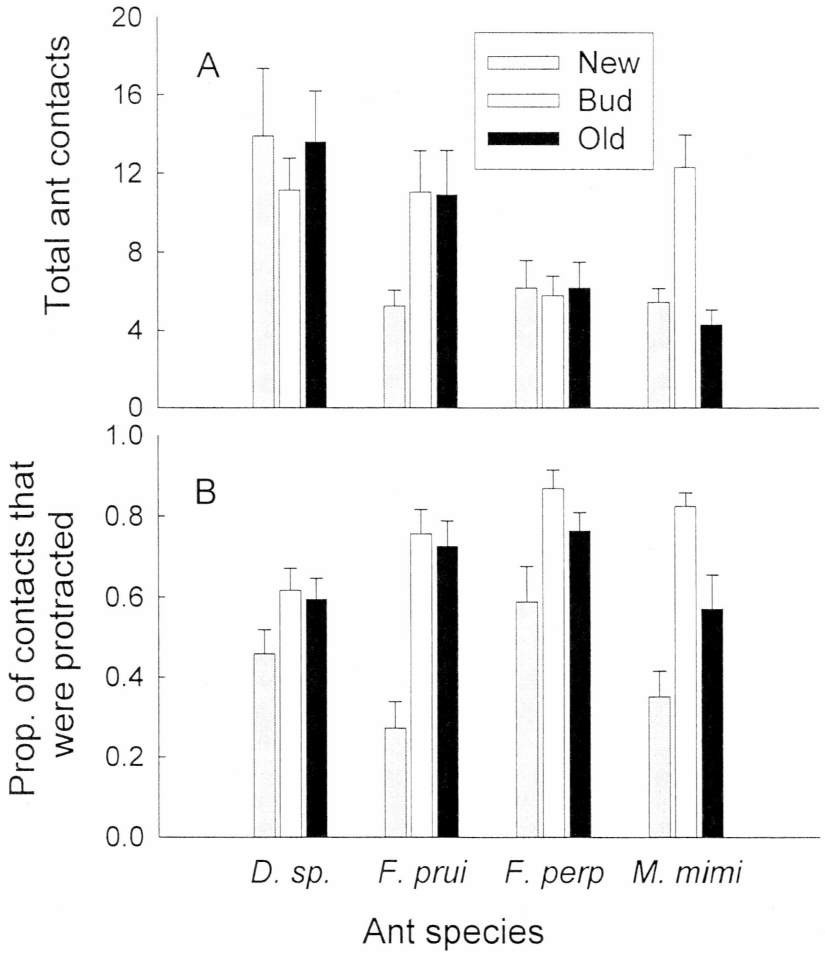

Figure 2.3: Total and protracted ant contacts to floral stages. A. Mean number of ant contacts to floral stages (including explorative and protracted contacts) for each ant species. B. Mean proportion of contacts to floral stages for each ant species that were protracted. Error bars are standard errors.

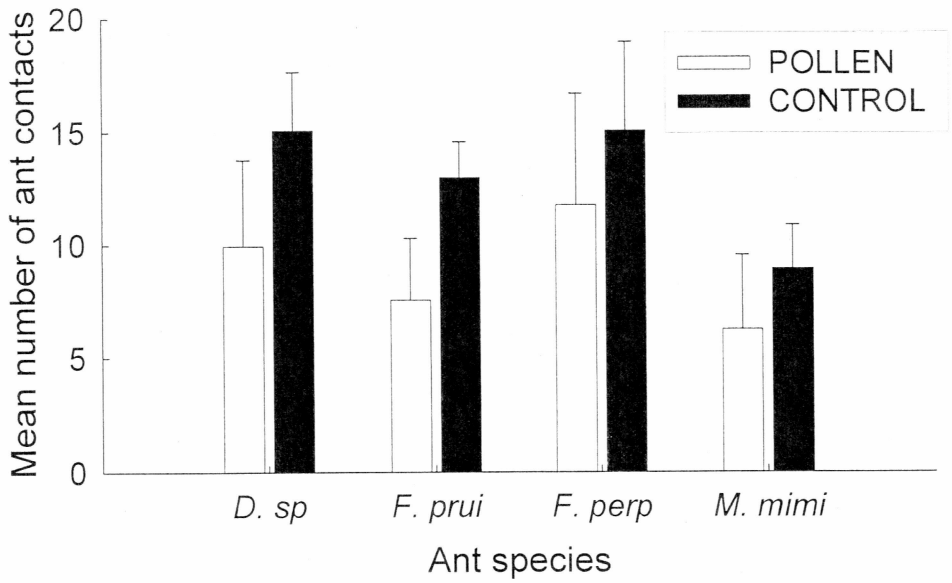

Figure 2.4: Pollen as an effective ant repellent. Mean (± 1 SE) number of ant contacts to pollen and control agar.

Conclusion

Past research has found both positive and negative impacts of ant visitation to *A. constricta*, suggesting that the interaction may be more antagonistic than mutualistic. There was no evidence that ants defended plants (Wagner 1997), and the effect of ants on male and female function appeared to conflict. Increased seed set correlated with ant-enriched soil nutrients (Wagner 1997), but was accompanied by reduced pollen viability (Wagner 2000).

I have shown that ants that increase soil nutrients at the base of *A. constricta* may actually contribute to plant defense in a way not previously considered. In a greenhouse study, increased soil nutrients resulted in greater allocation to chemical defenses. Additionally, my results suggest that plants with ant-enriched soils may produce more ant attractants in the form of EFNs, which encourage increased ant visitation and nesting and may serve to perpetuate a positive feedback cycle between ants and *A. constricta*.

Finally, I have shown that *A. constricta* utilizes several mechanisms to reduce the costs of ant visitation. Plants separate ants from pollen and pollinators by releasing pollen during the time of day ants are least active and by emitting a floral ant repellent associated with pollen. Given the novel benefits *A. constricta* receives from ants and the mechanisms plants have to minimize the potential costs of ant visitation, the relationship between *A. constricta* and ants appears to be more mutualistic than antagonistic.

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